CHEMOPROTECTIVE EFFECTS OF FLAXSEED LIGNANS ENTERODIOL AND ENTEROLACTONE IN NON-TRANSFORMED COLONOCYTES

A Thesis

by

CHRISTINA ALISON CURRY

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Chair of Committee,	Clinton Allred
Committee Members,	Joseph Awika
	Jenna Anding
Head of Department,	Boon Chew

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ABSTRACT

Previous epidemiological studies have shown that colon cancer incidence is correlated to diet and estrogen status. Phytoestrogens are molecules with similar structures to estrogen that occur naturally in plants. There is *in vitro* and *in vivo* evidence that phytoestrogens in the diet can inhibit carcinogenesis. The phytoestrogenic mammalian lignans enterolactone (EL) and enterodiol (ED) in flaxseed have been shown to be effective in decreasing tumor incidence in carcinogenic models, but there is little data regarding their effects in non-malignant cells. The following studies used a non-transformed cell line of young adult mouse colonocytes (YAMC) to determine the protective effects of ED and EL in chemoprevention.

Our results demonstrate that low levels of EL (1μM) and ED (5μM) are effective at significantly reducing cell growth and increasing apoptosis. These treatments also regulated transcription via significant differences in gene levels related to apoptosis and cell cycle progression. The data collected demonstrate some of the physiological effects of EL and ED on the cellular and molecular level. These changes may contribute to the overall effect of prevention of colon cancer carcinogenesis seen with flaxseed consumption.

DEDICATION

To Raph and Dandy,

For loving and supporting me unconditionally,

and allowing me to make my dreams a reality.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Allred for giving me this opportunity, and for his encouragement and guidance throughout. I would also like to thank my committee members Dr. Awika and Dr. Anding for their time and effort in helping me to complete my degree.

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Finally, I would like to thank my friends and family. To Mom, Dad and Claire, thank you for your constant support. I know I haven't always made it easy, but I couldn't have done it without you all. Abigail, thank you for being a shoulder to lean on, and an ear to bend throughout this whole process. You kept my spirits high, and kept me motivated to finish.

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CHAPTER I

INTRODUCTION

Colorectal cancer is predicted to be the third most common cancer in both men and women in 2015 with over 90,000 new cases in the United States.[1] Collectively, the literature shows a definitive link between estrogen receptors and colorectal carcinogenesis, and previous data from our laboratory has shown that estradiol (E_2) alters normal colonocyte growth and is protective against the formation of preneoplastic lesions.[2,3] These studies also revealed that the activity of E_2 in the colon occurs primarily through estrogen receptor beta (ERβ). Premenopausal women are less likely to develop colon cancer, and use of hormone replacement therapy (HRT) in postmenopausal women has been beneficial in reducing colon cancer risk. [4] Conversely, use of HRT post-menopause may also increase risk of breast cancer. [5] Because of this risk, dietary phytoestrogens have been targeted as a safer alternative for chemoprevention.

Phytoestrogens, and consumption of them over time have been shown to inhibit carcinogenesis in *in vitro, in vivo,* and in epigenetic studies. [6-7] Flax (*Linum usitatissimum)* contains a high amount of phytoestrogens in the form of lignans. Lignans are classified as non-flavonoid phytoestrogens and have been shown to have estrogenic effects. [8] The major lignan in flaxseed is secoisolariciresinoldiglucoside (SDG), along with other minor lignans including secosisolariciresinol and matairesinol. [9] When flaxseed is consumed by mammals, SDG is converted into enterodiol (ED) and enterolactone (EL) by gut bacteria. [10] Several *in vitro* and *in vivo* studies have demonstrated the anti-carcinogenic effects of flaxseed and its lignans. In the 2006 study

by Bommareddy et al., Fischer rats were fed either corn meal or flaxseed meal supplemented diets for a week before a carcinogen was introduced. After 36 weeks, serum and the gastrointestinal tracts were collected and analyzed. The rats fed flaxseed meal showed significantly decreased colon tumor incidence, average tumor number and size compared to rats fed corn meal diet. [11] In a similar study by the same author, Apc^{Min} mice that were fed flaxseed supplemented diets showed significantly less tumor multiplicity and size in the colon and small intestine as compared to the corn supplemented control group.[12] Bommareddy, Sung, and Danbara have all demonstrated inhibition of tumor cell growth by flax lignans EL and ED using human colon cancer cell lines. [13, 14, 15]

Currently, the *in vivo* research concerning ED and EL in colon carcinogenesis is exclusively presented in transformed carcinogenic cell models. The present experiments were completed in non-transformed mouse colonocytes to better understand the protective mechanisms pre-carcinogenesis. As colorectal cancer is predicted to be the third most prevalent cancer in the United States in the year 2015, the focus of anti-cancer research has shifted to prevention through lifestyle modifications. The lignans from flaxseed have been shown to be effective in preventing tumorigenesis *in vivo*, and could possibly offer a safer alternative to HRT in preventing colon cancer.

We hypothesized that the flaxseed lignans EL and ED will decrease cell growth, and alter cell morphology of the colonocytes via modulation of transcription regulators. These effects will confer protection against the development of colon cancer. To test our hypothesis we used a non-transformed young adult mouse colonocyte (YAMC) cell line.

CHAPTER II

LITERATURE REVIEW

Research Justification

Colon Cancer

The colonic epithelium is constructed of crypts increasing the surface area available for absorption as well as separating the lumen from the lamina propria. There are different types of cells organized in layers within the crypt. Stem cells located at the base of the crypt proliferate rapidly differentiating into transit amplifying (TA) cells and eventually enterocytes; pushing existing cells up the crypt, and ultimately shedding them via apoptosis, or programmed cell death.[16] Like most epithelial tissue, the sloughing off of dead cells near the luminal end of the colonic crypt is integral to the health of the tissue and is a tightly controlled process. Apoptosis is mediated either extrinsically following binding of ligands to cell surface receptors or intrinsically from DNA damage or oxidative stress. In both cases the Caspase-3 protein is produced in the cell, signaling the final events of cell death.[17] Carcinogenesis occurs when this cycle is disrupted and a polyp is formed in the crypt, which forms into an adenoma and eventually metastasizes.[18] Originally, it was thought that cell proliferation was a critical marker for tumorigenesis; it is now accepted that apoptotic activity, specifically the inhibition of apoptosis, is an important indicator of tumor progression.[19,20]

Colon cancer is the second most common cause of cancer death, and is primarily seen in Caucasians with northern European heritage. It is less commonly seen in those of Asian and African descent, but incidence rises with westernization and migration.[21]

Generally, adult men are more likely to develop colon cancer at an earlier age than women, but the risk for women increases greatly after menopause when levels of endogenously produced estrogen drop dramatically.

Estrogen

Estrogens are cholesterol derived steroid hormones that are produced endogenously and are integral to homeostatic regulation in both males and females. Though typically linked to carcinogenesis in reproductive tissues, estrogen is also linked to other forms of cancer development in lung and gastrointestinal system tissues including the colon. There are two different estrogen receptors (ER) in the body, alpha (ERα) and beta (ERβ). When activated, ERα promotes proliferation while ERβ generally induces anti-proliferative effects via apoptosis.[22] The ERs are tissue specific with ERβ being the primary receptor in the colonic tissue.[23] Because of ERβ's prevalence in the colon, it has been implicated in the development and progression of colon cancer. Expression of ERβ in colon tissue is decreased with tumorigenesis and is inversely related to progression of colon cancer.[24,25] Estrogen signaling in the colon has also been studied as an important mechanism in protecting against carcinogenesis in the colon.[26]

Previous studies from our laboratory have demonstrated treatment of non-malignant cells with 17β-estradiol (E_2) *in vivo* and *in vitro* increases apoptosis and decreases cell growth in a dose-responsive pattern. This process is is mediated by ERβ.[3] Briefly, young adult mouse colonocyte (YAMCs) treated with 100-10,000pmol E_2 exhibited a significant decrease in cell growth as well as a significant increase in

apoptosis measured by the caspase-3 protein. Female heterozygous estrogen receptor beta knockout (ERβ-KO) and wild type (WT) mice were utilized in the *in vivo* study to better understand the necessity of ERβ for estrogenic chemoprotection. Mice were ovariectamized to prevent endogenous estrogen production and a cholesterol or 1nm estradiol pellet was inserted subcutaneously. Azoxymethane (AOM), a chemical carcinogen, was injected and mice were sacrificed at eight weeks later. At the time of sacrifice, blood and colonic tissue were resected and preserved for further analysis. WT animals treated with E_2 saw significantly fewer aberrant crypt foci (ACF), which are preneoplastic lesions indicative of carcinogenesis. This effect was almost completely lost in the ERβ-KO mice, indicating the need of a functional ERβ for E_2 to incur protective effects against colon cancer.

Use of postmenopausal hormones has been shown to decrease the incidence of colon cancer in postmenopausal women by approximately 30-40%.[27] Unfortunately, epidemiological evidence suggests that while lowering colon cancer risk, hormonal replacement therapy may also increase risk of developing breast cancer within five years of use.[5] Due to this risk, interest in dietary compounds for the prevention of colon cancer has increased.

Phytoestrogens

Phytoestrogens are naturally occurring compounds in plants that are structurally similar to the human hormone estradiol. These compounds have the ability to cause estrogenic or anti-estrogenic effects. Types of phytoestrogens include isoflavones,

coumestanes, and phytoestrogen precursors called plant lignans which are transformed by the gut microbe of mammals into bioactive estrogenic mammalian lignans. In Asian countries where diets are plant-based and high in phytochemicals, mortalities of breast, prostate, and colorectal cancer are lower than those in the western world where diets higher in animal proteins and saturated fats alter the production and function of steroid hormones.[28]

In vivo studies from our laboratory suggest genistein (GEN), the primary phytoestrogen found in soybeans, has chemo-protective functions in the colon of mice.[29] Thirty mice were ovariectamized and either an E_2 or cholesterol pellet was inserted subcutaneously at the base of the neck on the back. One group of cholesterol treated mice were given a powdered GEN diet for 28 days before sacrifice and compared to mice treated with 1nm E_2 or cholesterol alone. Colon tissue was collected and the distal portion was sectioned and preserved in formaldehyde for analysis. Using a TUNEL assay it was observed that colonic tissue from mice treated with E_2 displayed a greater amount of apoptotic cells compared to the control. Those mice fed the GEN diet also saw an increase in apoptosis, but not at the same level as E_2. This study not only demonstrated that E_2 does induce the protective mechanism of apoptosis in the colonic crypt but that the phytoestrogen genistein may be beneficial in chemo-protection of the colon as well .

Other phytoestrogens have also been effective chemo-protectants as evidenced in *in vitro* studies. Phytoestrogens trigonelline (Trig) and diindolylmethane (DIM) have demonstrated regulation of YAMC cell growth via apoptosis and disruption of the cell

cycle.[30] These effects were found to be mediated by ERβ, though neither compound bound directly to the receptor-binding pocket of the protein.

Flaxseed

Flax (*Linum usitassimum)* is a flowering herb that belongs to the Lineaceae family. Native to West Asia and the Mediterranean, it has been grown and harvested since ancient times and is now cultivated around the globe with Canada, India, China, and Ethiopia being some of the largest exporters.[31,32] Its seeds, also known as linseeds, are small and flat with a nutty taste.[31] They have a seed coat called a testa, two embryos that make up most of the seed by weight, and a thin endosperm.[33] The fiber from the flax plant can be used to create threads, twine and fabrics while the seeds are consumed for their oils. When consumed flax is referred to as flaxseed while linseed is used to describe flax used in textiles and other industrial purposes.[34]

Flaxseed is favorable for consumption due to its high levels of alpha-linolenic acid (ALA), fiber, and lignans, specifically SDG. ALA is an essential omega-3 fatty acid that makes up over 50% of the lipid content in flaxseeds.[35] Omega-3 fatty acids (ω-3 FAs) are essential lipids that have been shown to be beneficial in the treatment and mitigation of several conditions. Essential fatty acids are crucial to the normal functioning of cell membranes, organs like brain, kidney and liver, and are a key component of hormone functioning. ω-3 FAs have also been shown to help prevent and mitigate chronic diseases such as cardiovascular disease, diabetes, obesity, metabolic syndrome, and gastrointestinal disorders.[36] Studies also suggest ω-3 FAs may have anti-

proliferative effects in cancer cells and may have the ability to improve the quality of life of cancer patients.[37] The 2010 Guidelines for Healthy Americans recommend consuming at least 8 ounces of fish a week as a source for approximately 250mg of the ω-3 FAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[38] Flaxseed provides an alternative source of ω-3 FAs for those who cannot or choose not to eat fish, or who do not eat enough fish to meet this requirement.

The fiber from flaxseeds also contributes health benefits. Consuming dietary fiber can lower blood glucose levels by slowing gastric emptying and the release of sugar into the blood.[39] Consuming high amounts of dietary fiber has also been shown to prevent weight gain in both men and women.[40] There are approximately 25g of dietary fiber per 100g of flaxseed.[41] The fiber in the seed is located in the outer layer known as the mucilage which has a high water-binding capacity. This water-binding trait helps to alleviate and prevent constipation.[39]

Flaxseeds also contain a high amount of phytoestrogens in the form of lignans. Lignans are classified as non-flavonoid phytoestrogens and have been shown to have estrogenic effects.[8] Flaxseeds contain SDG, the major lignan, as well as minor amounts of the lignans secosisolariciresinol and matairesinol.[9] When mammals consume plant lignans they are transformed by the gut microbes in the colon into bioactive compounds. It is believed that a group of microorganisms in the colon are responsible for the transformation of SDG to the mammalian lignans enterodiol (ED) and enterolactone (EL). Wang et al. identified five different genetic bacterial lineages involved in this transformation via gel electrophoresis and named the consortium END-49.[42] Clavel and

Woting were able to define the species responsible for transformation as *Clostridium saccharogumia*, *Eggerthella lenta*, *Blautia producta*, and *Lactonifactor longoviformis*[43,44]. Collectively these microorganisms are capable of converting SDG into EL and EL via secosisolariciresinol (Seco).

Plasma levels of ED and EL in humans are inversely correlated with colon cancer incidence. The Diet, Cancer and Health cohort conducted in Denmark followed over 50,000 participants aged 50-64 for almost six years and a random selection of the cohort was selected for analysis of their plasma for EL levels. For women, a higher concentration of EL in the plasma was associated with a lower risk for colon cancer. Interestingly, for men, an increase in plasma EL was associated with a higher risk of colon cancer.[45] Kujisten et al. saw similar results with both lignans. The researchers used data from the retrospective case-control POLIEP study conducted in the Netherlands. Participants in the study were aged 18-75 with no history of inflammatory bowel disease or colon cancer. Each participant underwent an endoscopy, completed a questionnaire about their diet, medical history, and lifestyle, and gave a plasma sample. Participants with higher levels of both ED and EL in their plasma showed reduced risk of colorectal adenomas in a dose-dependent manner.[46]

Several *in vivo* studies performed in mice and rats have shown similar protective effects. One study compared flaxseed oil diets to those of corn oil and the effects on colon tumor development in Fischer rats.[47] The carcinogen AOM was injected once a week for three weeks before the rats were sacrificed and blood and colonic tissue were collected. There was 100% tumor incidence in the corn oil group as compared to only

54% in those fed flaxseed oil. Tumor size and multiplicity were also significantly larger in the corn oil group when compared to the flaxseed oil group. Bommareddy et al. conducted a similar experiment during which they fed their male Fischer rats either flaxseed or cornmeal diets, injected AOM, and collected blood and colon tissue samples.[11] They also saw decreased tumor incidence in the flaxseed meal group. Using western blots they discovered that the COX-1 and COX-2 proteins were significantly lowered in the colonic tissue of the flaxseed meal diet group. High levels of the COX protein in the cornmeal group may have propagated tumor formation because of the protein's role in the conversion of arachidonic acid to prostaglandins which are known tumor promoters. Another similar study compared flaxseed meal to flaxseed oil diets in chemoprevention.[48] Fischer rats were fed either flaxseed meal or flaxseed oil and AOM was introduced at seven and eight weeks. Both flaxseed oil and flaxseed meal were effective in reducing the incidence of ACF in both the proximal and distal colon. These results were also confirmed in the Apc^{Min} mouse model, which have point mutation in the Apc gene, leading to the development of polyps in the colon. The results suggested that dietary flaxseed could protect against tumor formation, evidenced by significantly decreased tumor multiplicity in the mice fed flaxseed meal or oil.[12] Hernandez-Salazar examined flaxseed's influence on gene expression involved in colon carcinogenesis.[49] They fed Sprague-Dawley rats flaxseed and induced carcinogenesis via AOM injections. Those mice fed flaxseed diets had decreased crypt multiplicity of ACFs and modulated the expression of cell cycle arrest and apoptotic genes: *p53*, *p21*, *bcl-2*, *bax*, and *caspase-3*.

In order to better elucidate cellular mechanisms of flaxseed, several *in vivo* experiments were conducted. Researchers used the Caco-2 cell line to model colon epithelial adenocarcinoma. After treating cells with EL and ED, BrdU and propidium iodine staining were used to analyze proliferation and apoptosis respectively. Both EL and ED caused a significant increase in apoptotic cells and a decrease in proliferation.[13] Similar results were seen with EL in the Colo 201 human tumor cell line.[15] So far, the majority of the *in vitro* research regarding the chemoprotective effects of EL an ED has been completed in transformed cell lines. More research is needed to better understand the mechanisms of protection prior to carcinogenesis.

Specific Aims

To determine the effect of flaxseed lignans (enterolactone) EL and (enterodiol) ED on cell growth, maintenance, and apoptosis in non-malignant colonocytes.

Hypothesis

We predict:

1. Non-malignant colonocytes treated with ED or EL will show similar chemoprotective effects to those treated with E_2.
2. Treated cells will show decreased growth and increased apoptosis via transcriptional regulation.

CHAPTER III

CHEMOPROTECTIVE EFFECTS OF FLAXSEED LIGNANS ENTERODIOL AND ENTEROLACTONE IN NON-TRANSFORMED COLONOCYTES

Introduction

With colon cancer predicted to be the third most common cancer in 2015, there has been an increased research emphasis on prevention of colon cancer.[1] Typically, pre-menopausal women are less likely to develop colon cancer when compared to their post-menopausal counterparts. The Women's Health Initiative Study recognized that use of HRT in postmenopausal women was correlated to a significant decrease in the incidence of colon cancer.[50] Estrogen's chemoprotective mechanisms have been studied extensively, and previous studies from our laboratory have demonstrated these effects both *in vivo* and *in vitro*.[3,26,29,30,2]

Recently, there has been an increased interest in the role of diet in cancer prevention. Because colon cancer, and protection against colon cancers, is estrogen-mediated, phytoestrogens have been studied as an alternative to HRT for chemoprevention.[6,51] Common phytoestrogens found in plant based diets include GEN from soy and the mammalian lignans EL and ED that are formed when flaxseed is consumed by mammals and processed by the gut microbe.[52] Previous research from our laboratory has proven the effectiveness of GEN, as well as other phytoestrogens like Trig and DIM, in chemoprotection of non-transformed colonocytes.[29,30]

Research has shown that consumption of flaxseed is linked to lower risk of colon cancer development.[53] Both *in vitro* and *in vivo* studies have provided possible

mechanisms of action, mainly mediated through the prominent ER in the colon, ERβ.[13,24] The majority of the studies have focused on carcinogenesis models, with little to no emphasis on estrogenic effects pre-transformation.

Our goal was to identify possible mechanisms by which the flaxseed lignans EL and ED may confer protection against the development of colon cancer, primarily through apoptosis and cell signaling pathways.

Materials and Methods

Reagents

Enterodiol (ED) and enterolactone (EL) were purchased from Sigma-Aldrich. Reagents were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich).

Cells

Young Adult Mouse Colonocytes (YAMC) were obtained from Dr. Harmut Land (University of Rochester Medical Center). The cells are derived from the Immorto-mouse and express the temperature-sensitive simian virus 40 large T under the control of an interferon-γ inducible promoter. The cells were maintained in permissive conditions, 33°C, 5% CO_2 with 5 units interferon-γ (IFN-γ; Roche), and cultured in RPMI1640 (Sigma Aldrich) with 10% fetal bovine serum (FBS; Hyclone), 0.1% insulin, transferrin, and selenious acid (ITS; BD Biosciences) and 1% gentamicin (GIBCO) on collagen coated plates. For experiments cells were transferred to media containing 10% charcoal-dextran stripped FBS, 0.1% ITS and 1% gentamicin 48 hours before conducting

experiment. During experiments, cells were placed at non-permissive conditions; 39°C, 5% CO_2.

Cell Growth Assay

Cells were seeded at a concentration of 3.0 x 10^4 cells/well in 6-well collagen coated plates (Grenier bio-one), and grown at the permissive condition with IFN-γ for 24 hours. The cells were treated with ED or EL at concentrations of 1, 5,10 μM, and 100nM or 0.1% DMSO and transferred to non-permissive conditions for 96 hours. Media with treatments were replaced after 48 hours. After 96 hours, cells were trypsinized and collected for counting. Cell numbers were counted using a Cellometer Auto 1000 cell counter (Nexcelom). There were three wells per treatment, and each well was counted in triplicate. The experiment was replicated three times.

Caspase-3 Activity

Cells were seeded and grown in same conditions as the cell growth assay. Cells were treated for 96 hours with 1nM E_2, 5μM ED, 1μM EL, and 0.1% DMSO. After 96 hours of treatment, cells were trypsinized, collected and twice washed with PBS. Caspase-3 activity was measured according to the manufacturer's protocol of EnzChek Caspase-3 assay kit #2, Z-DEVD-R110 substrate (Molecular Probes). Cells were lysed and centrifuged, and supernatants transferred to a 96-well plate (BD Bioscience). 50μL of 2X working solution was added to each sample and incubated for 30 minutes in the

dark before reading. Fluorescence was measured with 496 (excitation)/ 520 (emission) nm at 15 minute intervals using a CLARIOstar microplate reader (BMG).

Flow Cytometry

Cells were seeded and grown in the same conditions and treatment as stated previously in the Caspase 3 assay. Cells were trypsinized and collected, and the supernatant was removed. After, they were washed with ice-cold 1X Phosphate Buffer Saline (PBS) and fixed overnight with 100% ethanol at -20°C. The cells were then stained with propidium iodide staining solution (50µg/ml propidiun iodide, 200µg/ml DNase-free Rnase, 5mM sodium citrate and 0.1% Trition X-100) at room temperature for 30 min. Using an Accuri C6 flow cytometer (BD Bioscience), 2 x 10^4 live cells were analyzed for cell cycle distribution.

RNA Extraction

Cells were seeded at 10 x 10^4 cells/well in 6-well plates and grown at permissive conditions with IFN-γ for 24 hours. The cells were then treated with 1nM E_2, 5µM ED, 1µM EL or 0.1% DMSO for 24 hours. RNA was isolated using the manufacturer's protocol for TRIzol Reagent (Invitrogen). Cells were trypsinized and collected, then one mL TRIzol reagent was added to lyse the cell membrane. 200µL of chloroform was added and the aqueous phase was collected. 500µL isopropanol was added to precipitate the RNA, and the pellet was washed with 75% ethanol, air dried, then dissolved in Rnase-free water. Rnase were inactivated at 55-60°C and then stored at -70°C.

Real Time PCR

The Transcriptor First Strand cDNA (Roche) synthesis kit was used to synthesize cDNA, and the manufacturer's protocol was used. 1µg of RNA plus 1µL oligo dT primer and 2µL random Hexomer primer were incubated at 65°C for 10 minutes. Next, 4µL reaction buffer, 0.5 µL Rnase inhibitor, 0.5 µL reverse transcriptase, and 2 µL dNTP were added and incubated at 25°C for 10 minutes, 50°C for 60 minutes, and 85°C for 5 minutes. cDNA was stored at -20°C.

Real time PCR was completed using the FastStart Universal SYBR Green Master mix (Roche). The primers used and their sequences are as follows: Bcl-2 (Forward: ATC TTC TCC TTC CAG CCT GA, Reverse: TCA GTC ATC CAC AGG GCG AT), c-Myb (Forward: TGT CAA CAG AGA ACG GC TGA, Reverse: CAC AGA ACC ACA CTT GCA GC), c-Myc (Forward: GCC CSG TGA GGA TAT CTG GA, Reverse: ATC GCA GAT GAA GCT CTG G), Mdm2 (Forward: TGT CTG TGT CTA CCG AGG GTG, Reverse: TCC AAC GGA CTT TAA CAA CTT CA), CCND1 (Forward: GCG TAC CCT GAC ACC AAT CTC, Reverse: ACT TGA AGT AAG ATA CGG AGG GC), pS2 (Forward: AGC ACA AGG TGA TCT GTG TCC, Reverse: GAA GCC AGA ATT TAT CCT CTC CC), and 18SRNA used as a control (Forward: TCA AGA ACG AAA GTC GA GGT, Reverse: GGA CAT CTA AGG GCA TCA CAG). Real time PCR was run on a Lightcycler® 480II (Roche) : 95°C for 10 minutes, 45 cycles of 15 seconds at 95°C and 30 seconds at 60°C.

Statistical Analysis

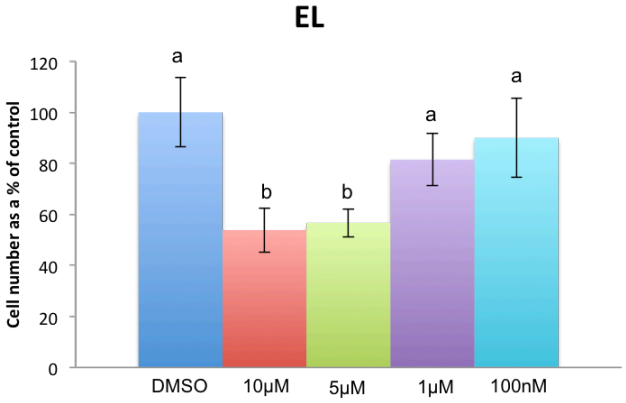
Each experiment was replicated in triplicate. The data is reported as the mean ± standard deviation (SD). Statistical significance was determined using JMP Pro 10 by a one way ANOVA, with $p<0.05$ considered significant.

Results

ED and EL Inhibit YAMC Cell Growth

In order to determine the effects of ED and EL on cell growth *in vitro*, YAMCs were treated with 10μM, 5μM, 1μM, and 100n EL and ED separately, with 0.1% DMSO as the vehicle control. Cells treated with EL displayed a dose-dependent decrease in growth. 10μM and 5μM concentrations of EL and ED were significantly different ($p<0.05$) when compared to control (Fig 2).

A.



B.

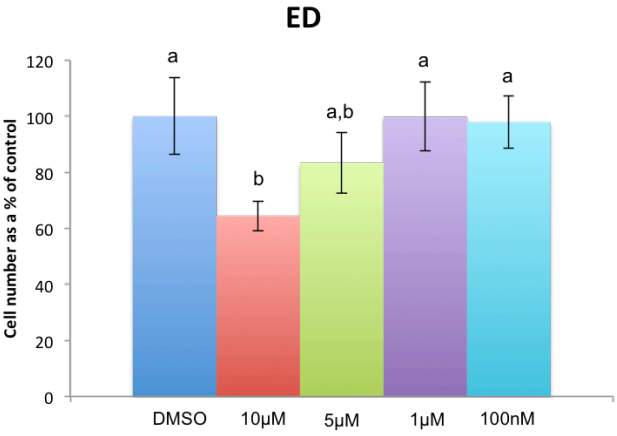
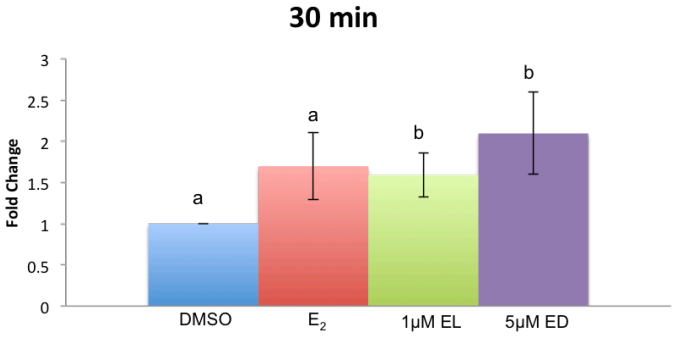


Fig. 1: EL and ED inhibit growth of YAMC cells. Cells were grown in charcoal dextran-stripped media for 48 h prior to plating. Cells were seeded at 30,000cells/well and grown at 39°C for 24 h prior to treatment. Cells were treated with 10μM, 5μM, 1μM, and 100nM EL, A and ED, B with 0.1% DMSO vehicle control for 96 h. Data are expressed as a percentage of the DMSO control group. Mean (n=9) +/− SEM from three replicate experiments. Bars without a common letter differ, $p < 0.05$.

Caspase-3 Activity in YAMC Cells Treated with ED and EL

After observing a decrease in cell growth of YAMCs following treatment with ED and EL, the next step was to determine the level of apoptosis in those cells. Caspase-3 activity was measured in cells treated with 1µM EL and 5µM ED with 0.1% DMSO as the vehicle control, and results were compared to the apoptotic activity of E_2. Fluorescence measurements were taken at 30, 45, and 60 minutes. The results suggest that there is a similar effect of caspase-3 activity in cells treated with ED and EL (Fig. 3) when compared to the positive control of E_2.

A.



B.

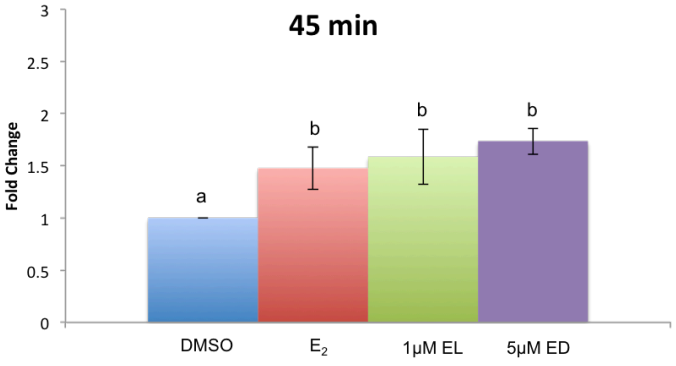


Fig. 2: Apoptosis in YAMCs treated with EL and ED. Cells were grown in charcoal dextran-stripped media for 48 h prior to plating. Cells were seeded at 30,000 cells/well and grown in 39°C for 24 h prior to treatment. Cells were treated with 5µM ED, 1µM EL, or 1nM E_2, with 0.1% DMSO vehicle control for 96 h. Fluorescence was measured at A. 30 minutes, B 45 minutes and C 60 minutes. Data are expressed as a percentage of the DMSO control group. Mean (n=9) +/− SEM from three replicate experiments. Bars without a common letter differ, $p < 0.05$.

C.

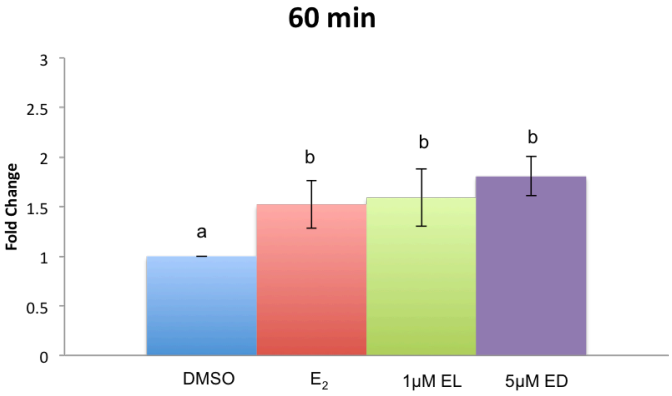


Fig 2: Continued

Cell Cycle Modulation by EL and ED

Our next objective was to test whether EL and ED could modulate progression of the cell cycle. The YAMCs were handled the same as the previous experiments and collected after 96 hours of treatment. The cells were then fixed, stained and analyzed for cell cycle stage via flow cytometry (Fig. 4). There was not a significant difference between the treatments in regulation of cell cycle.

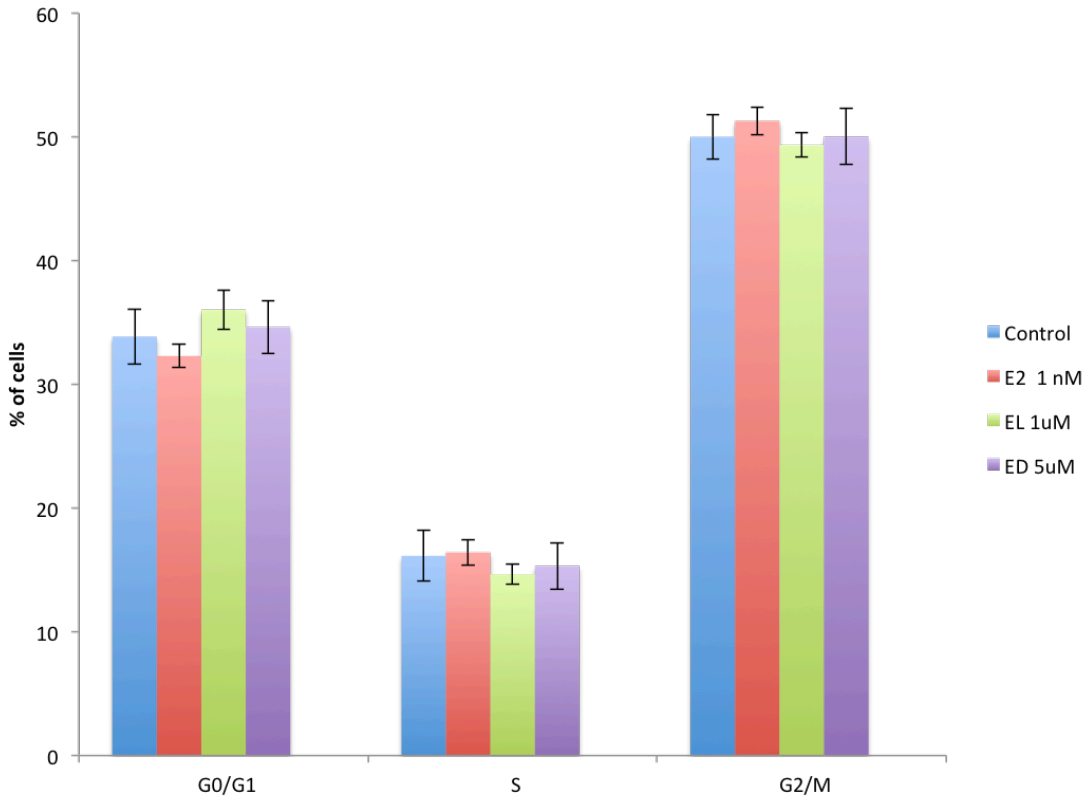


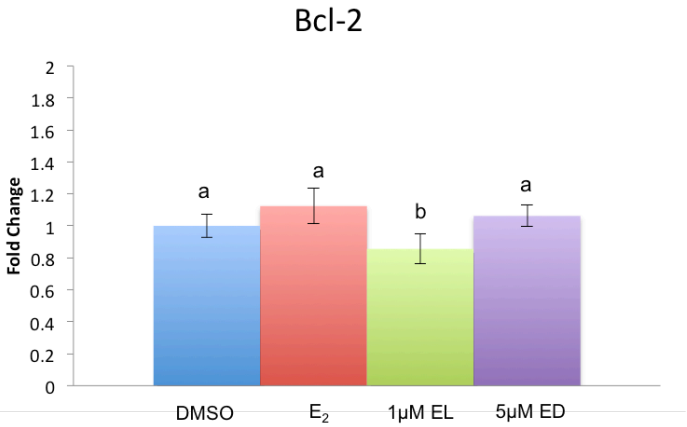
Fig. 3: Cell cycle modulation by EL and ED. Cells were grown in charcoal dextran-stripped media for 48 h prior to plating. Cells were seeded at 30,000 cells/well and grown in 39°C for 24 h prior to treatment. Cells were treated with 5μM ED, 1μM EL, or 1nM E_2, with 0.1% DMSO vehicle control for 96 h. Cells were fixed and stained with propidium iodine and then analyzed by a flow cytometer. Mean (n=9) +/− SEM from three replicate experiments.

Real Time PCR

The final experiment was to test if EL and ED were transcriptional regulators. Cells were seeded and treated with 5μM ED, 1μM EL, and 1nm E_2, with 0.1% DMSO vehicle control for only 24 hours. After cells were collected, RT-PCR was run with the gene targets Bcl-2, CCND1, Mdm2, and c-Myb, with 18s used for control (Fig. 5). Expression of Bcl-2 was significantly decreased by EL. Mdm2 expression was only

significantly reduced by E_2 treatment, and both EL and ED treatments were effective in significantly lowering expression of CCND1. Levels of c-Myb were decreased slightly, but not significantly by treatment with E_2, EL and ED.

A.



B.

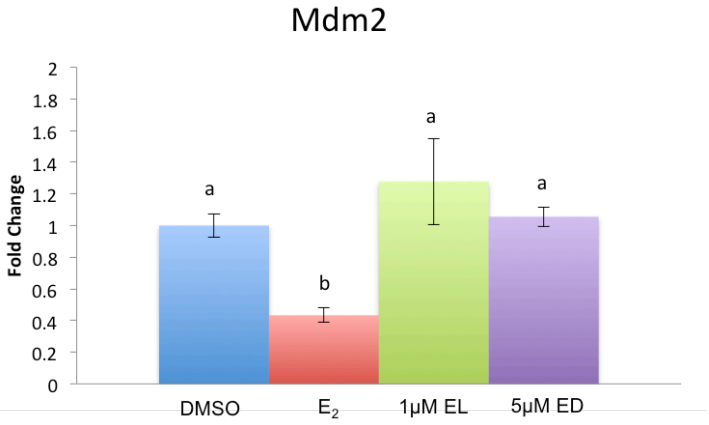
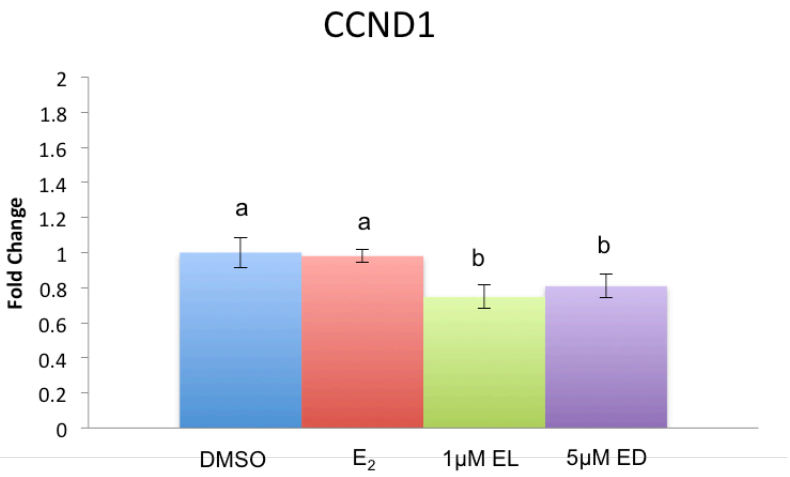


Fig. 4: Effect of EL and ED on gene expression. Cells were treated with 5μM ED, 1μM EL, and 1nM E_2, with a 0.1% DMSO control for 24 hours. Amplification was measured using SYBR green expression. A. Bcl-2, B. CCND1, C. Mdm2, and D. c-Myb. Gene expression was normalized by 18S. Values are mean (n=27) +/− SEM from three replicate experiments. Bars without a common letter differ, $p < 0.05$.

C.



D.

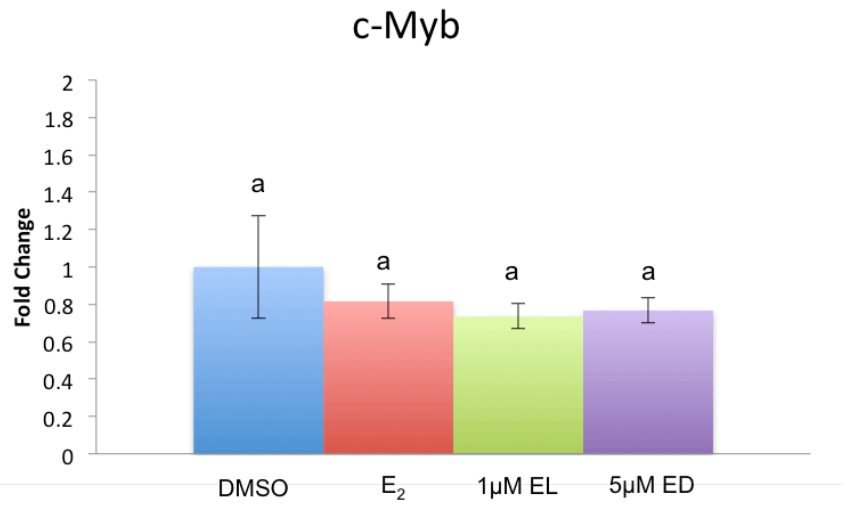


Fig. 4: Continued

Discussion

Flaxseed has been targeted for its many health benefits including its anti-cancerous effects. Though it contains other important nutrients, the mammalian lignans, EL and ED, formed by the gut microbe were of particular interest because of their phytoestrogenic properties. Previous studies have shown these lignans to be effective in cancer treatment *in vivo* and *in vitro*, though none of the studies conducted focused on pre-malignant mechanisms for chemoprotection.[11–13,15,47,48] Additionally, studies from our laboratory have shown estrogen and phytoestrogens to be protective against colon cancer development both *in vivo* and *in vitro*.[3,29,30] The purpose of this study was to determine if flaxseed lignans were successful chemoprotective agents in non-transformed colon epithelial cells, with a focus on regulation of cell cycle and apoptosis.

The first step was to determine if treatment of the YAMCs with EL and ED was effective in reducing cell growth. At 10μM and 5μM of EL and ED growth was reduced significantly up to 40% less than the control at the highest dosage. We chose to chose to use 1μM and 5μM of EL and ED respectively because these doses reduced cell growth appropriately 20%, which was similar to the effects of E_2 reported previously.[3,29,30] In studies conducted in cancerous cell lines concentrations of EL and ED ranged from 50-200μM.[13,15] Because our experiments were conducted in non-transformed colonocytes, a much lower dose was chosen to prohibit cell growth from being reduced too drastically. Once appropriate levels of decreased growth were determined, the ability of these compounds to induce apoptosis was investigated. Using the caspase-3 protein as a marker, a significant increase of apoptosis with E_2, EL and ED treatment was seen.

These results indicate that increasing apoptosis may be a mechanism for the chemoprotective effect of EL and ED in colon carcinogenesis. Bommareddy et al. also demonstrated significant effects in apoptosis in Caco-2 cells, but the concentrations used ranged from 75-150μM.[13] We demonstrated that significant apoptotic effects could be seen in non-transformed cells using considerably lower concentrations of treatment.

The genes chosen for RT PCR analysis were known targets of estrogen regulation. Bcl-2 was one of the first known regulators of apoptosis in mammals. High expression of the gene over long periods of time correlates with inhibition of apoptosis, allowing for tumor development.[54] Therefore, down regulation of Bcl-2 in the colon epithelium permits programmed cell death to continue, decreasing the likelihood of adenoma formation. Our results show a significant decrease in Bcl-2 expression when treated with 1μM EL. This was consistent with previously published results when even higher concentrations were used.[15] The murine double minutes 2 oncoprotein or Mdm2 has been shown to be a negative regulator of the p53 tumor suppressor, and is overexpressed in cancerous tissues including colonic adenomas.[55] We did not find significant effects in cells treated with EL or ED. A significant effect was seen with E_2 treatment, consistent with previously reported data.[30] Increased expression of c-Myb is associated with colon cancers, and when heterozygously knocked down, survival has been shown to improve.[56] In our experiments, c-Myb was not significantly reduced, but showed a decrease in all three treatment groups. Cyclin D1 (CCND1) is a tightly controlled cell cycle regulator, and disruption of its expression is common in cancer.[57] CCND1 accumulates in the cell during G1 and is degraded as the cell transitions into the

S phase. Overexpression of CCND1 has been shown to correlate to increased cancer risk.[58] Our experiments showed that EL and ED significantly lowered expression of CCND1 after treatment, suggesting a potential mechanism for chemoprevention. Additionally, this result was interesting because CCND1 is integral to cell cycle progression, yet there were no significant differences seen in flow cytometry. In the cytometry experiments cells were treated for 96 hours, as opposed to only 24 hours in the RT PCR assay. This leads us to believe that there may be other mechanisms involved in Ccnd1 expression past 24 hours.

In conclusion, the compounds EL and ED reduced growth and increased apoptosis of YAMC cells. There was also evidence of transcriptional regulation of apoptotic and cell cycle progression genes. Previously their effects had only been studied in carcinogenic cell lines or animal models, with much higher treatment doses. Our results lead us to believe that these effects are a component of the protective effect of flaxseed in the prevention of colon carcinogenesis.

CHAPTER IV

SUMMARY AND CONCLUSIONS

With almost 72,000 new cases of colon cancer expected for men, and close to 66,000 for women in 2015, it has become imperative to identify mechanisms of chemoprotection. The mammalian lignans EL and ED from flaxseed have been implicated in preventing the development of colon cancer. These studies have been completed primarily in carcinogenic cell lines and animal models, with very little understanding of the effects these lignans have on non-transformed colonic tissue. Our goal was to better elucidate these mechanisms. Our first step was to test if EL and ED could depress YAMC cell growth. Both EL and ED significantly reduced growth of the cells in a dose-responsive manner. An appropriate concentration of the compounds was then chosen based on the percent decrease in cell growth. 1μM EL and 5μM ED each conferred around a 20% reduction in cell growth, which is similar to the effect seen when treated with physiological levels of estradiol. The dosages were used throughout the rest of the experiments. Out next step was to determine levels of apoptosis by measuring the amount of caspase-3 present in the cells after 96 hours of treatment. EL and ED were able to significantly increase apoptosis, similar to E_2, suggesting that apoptosis may be a contributing mechanism in chemoprotection via EL and ED. After observing decreases in cell growth and increases in apoptosis, the question of whether EL and ED would cause significant changes in cell cycle progression was investigated. No significance was found between the treatments and control, but there were significant effects found when changes in gene expression were tested. Real Time PCR was used to

determine the transcriptional modifications made by EL and ED in YAMCs. Bcl-2 was lowered significantly by EL treatment, but not ED, and only E_2 significantly reduced expression of Mdm2. These results suggest that EL and ED treatment may regulate apoptosis through other avenues. Interestingly, we did see some significance in genes related to cell cycle progression, despite not seeing any changes in the cell cycle assay. CCND1 was significantly reduced and c-Myb expression reduced, but not significantly. This data leads us to believe there may be some transcriptional regulation of the cell cycle by EL and ED. The difference between the RT-PCR and cell cycle assays was time of treatment. The cells used in RT-PCR were treated for only 24 hours, while the cell cycle assay used cells treated for 96 hours. This timing of treatment may be the reason for the difference seen in the results of the two experiments.

When compared to previous studies, our study used a much lower concentration of EL and ED because we wanted to preserve the health of the colonic cells, as well as stay as close to physiological levels of the lignans as possible. To our knowledge, this is the first study that has demonstrated the effects of the flaxseed lignans EL and ED on a non-transformed cell line. The compounds reduced growth, and increased apoptosis of YAMC cells. There was also evidence of transcriptional regulation of apoptotic and cell cycle progression genes, leading us to believe that EL and ED have protective effects against the initiation and progression of colon cancer.

Future Research

These studies were only the first steps to better understand the chemoprotective effects of flaxseed lignans in non-malignant colonocytes. Further steps should be taken to better understand their mechanisms of action. Next steps would include determining the compounds' interaction with the main estrogen receptor in the colon epithelium, ERβ. Additionally, studies should be conducted looking into the combinatory effects of EL and ED. *In vitro* studies may also offer more insight into the chemoprotective roles.

NOMENCLATURE

E_2	Estradiol
ERβ	Estrogen receptor beta
HRT	Hormone replacement therapy
SDG	Secoisolariciresinoldiglucoside
ED	Enterodiol
EL	Enterolactone
YAMC	Young adult mouse colonocyte
TA	Transit Amplifying
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ-KO	Estrogen receptor beta knock out
WT	Wild type
ACF	Aberrant crypt foci
AOM	Azoxymethane
GEN	Genistein
Trig	Trigonelline
DIM	Diindolylmethane
ALA	Alpha-linolenic acid
ω-3 FA	Omega-3 fatty acids
EPA	Eicosapentaenoic acid

DHA	Docosahexaenoic acid
Seco	Secosisolariciresinol

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